Journal of Polymers and the Environment, Vol. 8, No. 3, July 2000 (© 2002)

Biodegradation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a Tropical Marine Bacterium, *Pseudoalteromonas* sp. NRRL B-30083*

Timothy D. Leathers,[1,4] Nadathur S. Govind,[2] and Richard V. Greene[3]

Pseudoalteromonas sp. NRRL B-30083 was isolated as the predominant PHBV-degrading organism from a tropical marine environment. In complex medium, the isolate grew well at temperatures between 23°C and 33°C, with an optimal doubling time of about 30 min. NaCl was required at concentrations between 0.2 N and 0.8 N. Optimal pH levels for growth were between pH 6.5 and pH 8.5. Liquid cultures grew modestly on PHBV as a sole carbon source under optimal conditions, although PHBV depolymerase activity was not detected.

KEY WORDS: Biodegradation; marine; polyhydroxyalkanoates; poly(3-hydroxybutyrate-co-3-hydroxyvalerate); *Pseudoalteromonas*.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural bacterial polyesters that have demonstrated potential as replacements for petroleum-derived thermoplastics [1–3]. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a particularly promising PHA copolymer and has been the subject of commercial interest. Although PHAs have had difficulty competing economically with conventional plastics, they are attractive for certain niche applications in which disposal is prohibitively expensive, impractical, or illegal. One such application may be for use on ships at sea. Disposal of plastics at sea is restricted by U.S. law and international treaty, particularly the International Convention for the Prevention of Pollution from Ships, 1973, as modified by the Protocol of 1978 relating thereto (MARPOL 73/78). Large passenger and military vessels are essentially floating cities. They generate enormous volumes of waste, of which plastic is a major component. Biodegradable plastics are, therefore, attractive for maritime operations.

Numerous studies have been conducted on the biodegradation of PHAs in terrestrial environments, and many terrestrial microorganisms have been characterized that degrade these compounds [1, 3, 4]. Conversely, little is actually known about the fate of PHAs in marine environments, and only a few species of marine bacteria, including *Acinetobacter johnsonii*, *Comamonas testosteroni*, *Flavobacterium johnsoniae*, *Vibrio ordalii*, and *Zoogloea ramigera*, have been identified as PHA-degraders [5–7]. In water samples from the Zeebrugge Harbor on

* Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[1] National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL.

[2] Department of Marine Sciences, University of Puerto Rico, Mayaguez, PR.

[3] Office of International Research Programs, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.

[4] To whom correspondence should be addressed at National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture 1815 N. University St., Peoria, IL 61604; Telephone (309) 681-6377; Fax: (309) 681-6427; email: leathetd@mail.ncaur.usda.gov

1566-2543/00/0700-0119/0 © 2002 Plenum Publishing Corporation

the North Sea, 34 of 44 PHA-degrading isolates were identified as *Pseudoalteromonas haloplanktis* [8, 9]. Although these isolates were not further characterized, this observation suggests that *Pseudoalteromonas* may play an important role in the degradation of PHAs in marine environments. In this communication, we describe a new PHBV-degrading species of *Pseudoalteromonas* from a tropical marine environment.

MATERIALS AND METHODS

Growth Media

Defined minimal medium was amended with either 0.3% PHBV or 2.0% glucose. PHBV (5% HV) was obtained from Monsanto Chemical Co. (St. Louis, MO). In order to aid dispersion of PHBV suspensions, defined medium (50 ml) containing PHBV was sonicated for 20 min in a 300 ml flask immersed in an ultrasonic water bath (35 KHz, 285 W). Minimal medium contained (per liter of artificial sea water) 1.0 g NH_4 Cl; 0.25 g HEPES, adjusted to pH 7.6. Artificial seawater contained (per liter of distilled water) 23.48 g NaCl; 10.61 g $MgCl_2 \cdot 6H_2O$; 3.92 g Na_2SO_4; 0.66 g KCl; 0.47 g $CaCl_2 \cdot 2H_2O$; 0.19 g $NaHCO_3$; 96 mg KBr; 26 mg H_3BO_3; 40 mg $SrCl_2 \cdot 6H_2O$, adjusted to pH 8.3. Minimal media were amended with 15 g agar per liter to generate solid media. Complex liquid medium contained, unless otherwise noted (per liter of distilled water): 24.0 g NaCl; 7.0 g $MgSO_4 \cdot 7H_2O$; 5.3 g $MgCl_2 \cdot 6H_2O$; 5.0 g Peptone (Difco, Detroit, MI); 1.0 g Yeast Extract (Difco): 0.7 g KCl; 0.1 g $CaCl_2$, adjusted to pH 7.5. For pH studies, complex liquid medium was amended with the following buffers at 20 mM: MES, pH 5.5; MES, pH 6.5; HEPES, pH 7.5; TAPS, pH 8.5; CHES, pH 9.0. Solid complex medium contained 15 g of agar per liter of reconstituted Marine Broth 2216 (both from Difco).

Culture Maintenance and Growth Conditions

Bacterial stock cultures were stored at −80°C in 40% glycerol. Working stocks were maintained on solid complex medium. Liquid culture preinocula (10 ml of complex medium) were inoculated from working stocks and incubated overnight in 50 ml flasks at 200 rpm and 28°C. Experimental cultures (50 ml) were inoculated to a calculated initial OD_{600} of 0.05 and incubated in 300 ml flasks at 200 rpm and 28°C unless otherwise noted.

PHBV Depolymerase Assay

PHBV depolymerase was assayed essentially as described by Kobayashi *et al.* [10]. PHBV was suspended at 0.3% in 50 mM Tris buffer, pH 7.5. The suspension was sonicated for 20 min in a 300 ml flask immersed in an ultrasonic water bath (35 KHz, 285 W) prior to dilution to 0.03% in the same buffer. Culture supernatant samples (0.1 ml) were added to 0.9 ml of the substrate suspension and incubated for 24 h at 30°C. Activity was measured as the decrease in OD_{650} against substrate buffer blanks. Cell pellets from 1.0 ml of culture were resuspended in 1.0 ml substrate and assayed similarly. One unit is defined as the activity resulting in a change of 1.0 OD_{650} per minute [10].

16S Ribosomal RNA Gene Sequence Analysis

DNA from *Pseudoalteromonas* sp. NRRL B-30083 was isolated as described by Ausubel *et al.* [11]. Primers for the amplification of the 16s rRNA gene were from positions 519 (forward: 5′CCAGCAGCCGCGGTAATA 3′) and 1492 (reverse: 5′GGTTACCTTGTTACGAC 3′) on the *Escherichia coli* numbering system, representing approximately two-thirds of this gene [12]. Primers were synthesized with a Beckman Oligo 1000 DNA synthesizer. Polymerase chain reaction was performed using the genomic DNA and the two primers for 30 cycles as follows: denaturation at 95°C for 2 min, annealing at 30°C for 2 min, and extension at 72°C for 3 min. The reaction products were cloned into pCR®2.1 (Invitrogen Inc., Carlsbad, CA), and plasmids were sequenced by the dideoxy chain terminator method using the ABI 377 DNA sequencer with the dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence data were compared with those in the GenBank database using the BLAST program [13].

RESULTS AND DISCUSSION

Isolation and Identification of PHBV-Degrading Bacterium

Extruded plastics composed of PHBV or PHBV–starch blends were placed in seawater at various sites near the coast of southwestern Puerto Rico [14]. Water samples taken at these sites contained from 80 to 3,000 CFU/ml that produced halos of clearing on solid medium containing PHBV as a sole carbon source [14]. A representative colony of the predominant PHBV-degrading organism was purified and was capable of substantial PHBV degradation on solid medium (Fig. 1).

The isolate was a gram-negative, motile bacillus. DNA was prepared from the bacterium and amplified by PCR, using 16S RNA-specific primers as described in Materials and Methods, providing a sequence of approxi-

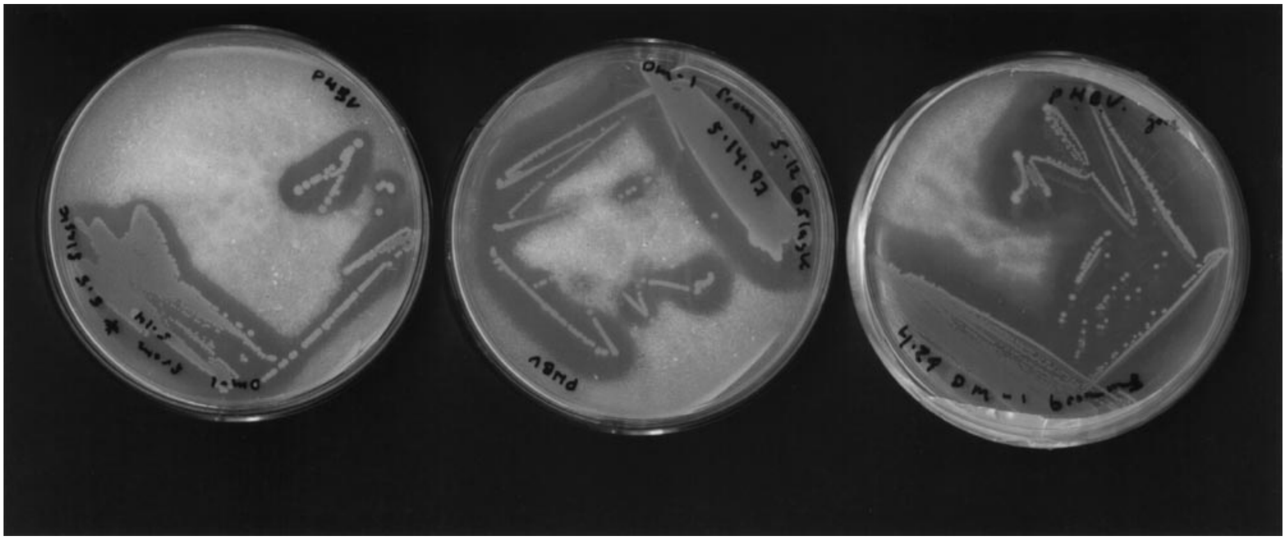

Fig. 1. Growth of *Pseudoalteromonas* sp. NRRL B-30083 on solid medium containing PHBV as a sole carbon source. PHBV degradation is indicated by clearing of the medium surrounding regions of growth.

mately one kb (Fig. 2). This sequence was submitted to GenBank (accession number AF317677). GenBank BLAST searches revealed that, at 97–99% sequence identities, the isolate is very likely a member of the genus *Pseudoalteromonas* (Table I). The isolate may or may not be conspecific with other strains at this level of similarity [15]. The isolate was accessioned by the ARS Patent Culture Collection (Peoria, IL) as *Pseudoalteromonas* sp. NRRL B-30083.

Optimal Growth Conditions

For determination of optimal growth conditions, *Pseudoalteromonas* sp. NRRL B-30083 was cultured on rich liquid medium as described in Materials and Methods. As previously reported, this strain was isolated from waters that averaged 26–32°C [14]. Consistent with this, the strain grew best at 28–33°C under laboratory conditions, although good growth was observed over a broad range of 18–38°C (Fig. 3). Interestingly, related strains include both thermophilic and psycrophilic isolates (Table I). NRRL B-30083 required salt for optimal growth (0.2 N to 0.8 N NaCl, Fig. 4) and grew best at initial pH values of 7.5–8.5 (Fig. 5), characteristic of a marine microorganism. Good growth was also observed at initial pH values in the broad range of 5.5–9.0, and culture growth resulted in a convergence of medium pH values toward the range of pH 7.0–8.0.

Growth and Enzyme Production on PHBV in Liquid Culture

Pseudoalteromonas sp. NRRL B-30083 was cultured under optimal growth conditions, as determined above, on minimal liquid medium containing either glucose or PHBV as a sole carbon source. Owing to the turbidity of cultures containing PHBV, growth was monitored by viable cell counts. Culture growth rates and yields were modest, as shown by the data in Fig. 6, and were roughly equivalent on either glucose or PHBV. However, PHBV depolymerase activity was not detectable ($<$0.01 U/ml) in culture supernatants and precipitates. Since the enzyme assay is very similar in principle to the solid medium screening method, this observation is perplexing, although not unprecedented. Saito *et al.* [7] found that 3 of 9 unidentified marine bacteria, isolated as PHBV degraders by solid medium screening, produced no measurable activity in assays of liquid cultures. To our knowledge, the only PHA depolymerase described from a marine bacterium is that from *Comamonas testosteroni* [6].

Pseudoalteromonas and Marine PHA Degradation

The genus *Pseudoalteromonas* was recently proposed as a component of a reorganization of the genus *Alteromonas* [16]. These gram-negative, motile bacteria have been isolated from diverse marine environments. *Pseudoalteromonas* isolates are often associated with higher marine organisms and have been found to produce agarases, novel polysaccharides, and a variety of bioactive compounds [17].

Few surveys have been undertaken that pertain to marine PHA degradation. Interestingly, however, the prominent degraders isolated from two rather different environments are closely related, if not the same, *Pseudo-*

```
  1 GGTTACCTTG TTACGACTTC ACCCCAGTCA TGAATCACTC CGTGGTGAAC GTCCTCCCGA
 61 AGGTTAGACT ATCCACTTCT GGAGCAACCC ACTCCCATGG TGTGACGGGC GGTGTGTACA
121 AGGCCCGGGA ACGTATTCAC CGCGTCATTC TGATACGCGA TTACTAGCGA TTCCGACTTC
181 ATGGAGTCGA GTTGCAGACT CCAATCCGGA CTACGACGCA CTTTAAGTGA TTCGCTTACC
241 CTCGCAGGTT CGCAGCACTC TGTATGCGCC ATTGTAGCAC GTGTGTAGCC CTACACGTAA
301 GGGCCATGAT GACTTGACGT CGTCCCCACC TTCCTCCGGT TTATCACCGG CAGTCTCCTT
361 AGAGTTCCCG ACCGAATCGC TGGCAACTAA NGATAGGGGT TGCGCTCGTT GCGGGACTTA
421 ACCCAACATC TCACAACACG AGCTGACGAC AGCCATGCAG CACCTGTATC AGAGTTCCCG
481 AAGGCACCAA ACCATTTCTG GTAAGTTCTC TGTATGTCAA GTGTAGGTAA GGTTCTTCGC
541 GTTGCATCGA ATTAAACCAC ATGCTCNACC GCTTGTGCGG GCCCCCGTCA ATTCATTTGA
601 GTTTTAACCT TGCGGCCGTA CTCCCCAGGC GGTNTACTTA ATGCGTTAGC TTTGAAAAAC
661 AAGTCCGAAG ACCCGAGCTT TTAGTAGACA TCGTTTACGG CGTGGACTAC CAGGGTATCT
721 AATCCTGTTT GCTCCCCACG CTTTCGTACA TGAGCGTCAG TGTTGCCCCA GGTGGCTGCC
781 TTCGCCATCG GTATTCCTTC AGATCTCTAC GCATTTCACC GCTACACCTG AAATTCTACC
841 ACCCTCTATC ACACTCTAGT TTGCCAGTTC GAAATGCAGT TCCCAGGTTG AGCCCGGGGC
901 TTTCACATCT CGCTTAACAA ACCGCCTGCG TACGCTTTAC GCCCAGTAAT TCCGATTAAC
961 GCTCGCACCC TCCGTATTAC CGCGGCTGCT GG
```

Fig. 2. Partial sequence of 16S RNA from *Pseudoalteromonas* sp. NRRL B-30083.

Table I. GenBank Database Identities with 16S RNA Sequence from *Pseudoalteromonas* sp. NRRL B-30083

Strain	GenBank accession no.	Identities	Gaps
Pseudoalteromonas sp. A28	AF227238	99% (987/992)	2/992
Pseudoalteromonas sp. S511-1	AB029824	99% (984/989)	2/989
Hydrothermal vent eubacterium PVB_OTU_5	HVU15114	99% (986/992)	2/992
Pseudoalteromonas sp. RE10F/5	AF118019	98% (971/990)	3/990
Pseudoalteromonas haloplanktis subsp. *tetraodonis* KMM	AF227237	97% (966/993)	3/993
Pseudoalteromonas sp. A25	AF214729	97% (972/992)	2/992
Pseudoalteromonas gracilis	AF038846	97% (964/990)	3/990
Psychrophilic marine bacterium PS39	AF200216	97% (963/990)	3/990
Pseudoalteromonas haloplanktis subsp. *tetraodonis* IAM	AF214730	97% (960/986)	3/986
Pseudoalteromonas sp. RE10F/2	AF118018	97% (963/990)	3/990

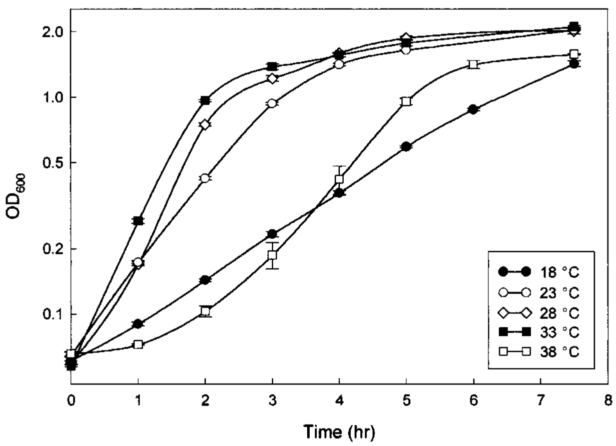

Fig. 3. Growth of *Pseudoalteromonas* sp. NRRL B-30083 in liquid complex medium at various temperatures.

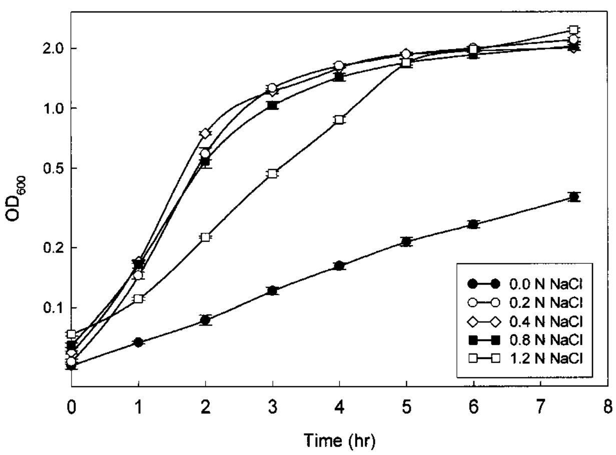

Fig. 4. Growth of *Pseudoalteromonas* sp. NRRL B-30083 in liquid complex medium containing various concentrations of NaCl.

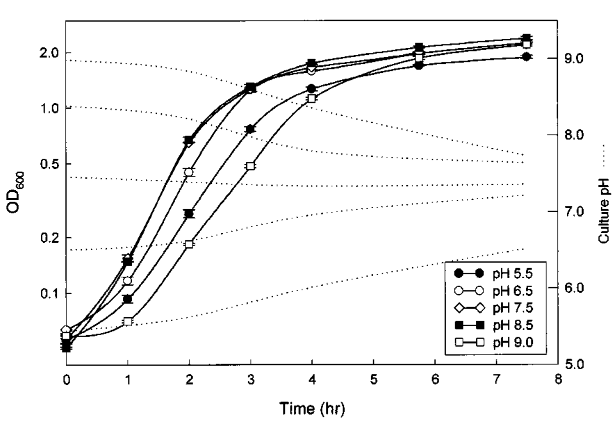

Fig. 5. Growth of *Pseudoalteromonas* sp. NRRL B-30083 in liquid complex medium at various pH levels.

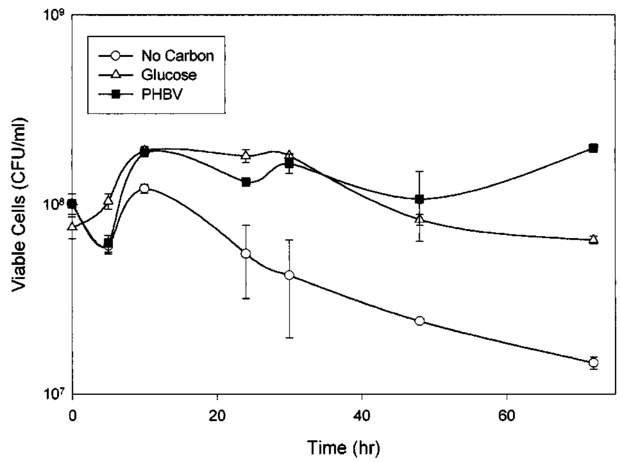

Fig. 6. Growth of *Pseudoalteromonas* sp. NRRL B-30083 in defined liquid medium containing no added carbon source, or either glucose or PHBV as a sole carbon source.

alteromonas species. The majority of PHBV-degrading isolates from the North Sea were assigned to the type species, *Pseudoalteromonas haloplanktis* [8]. No attempts to further characterize these isolates or their enzymes were reported. Similarly, *Pseudoalteromonas* sp. NRRL B-30083 was isolated as the prevalent PHBV-degrader from tropical waters near the coast of southwestern Puerto Rico. Although the strain produced distinct zones of clearing on solid medium containing PHBV as a sole carbon source, its poor growth and lack of PHBV depolymerase activity make it a difficult organism to fully characterize in liquid cultures grown on PHBV. Certainly, much remains to be learned about the action of marine microorganisms in general on PHAs. It is likely that *Pseudoalteromonas* species will play an important role in this process, however.

ACKNOWLEDGMENT

The authors are grateful for the expert technical contributions of Melinda S. Nunnally.

REFERENCES

1. H. Brandl, R. Bachofen, J. Mayer, and E. Wintermantel (1995) *Can. J. Microbiol.* **41,** 143–153.
2. H-M. Müller and D. Seebach (1993) *Angew. Chem. Int. Ed. Engl.* **32,** 477–502.
3. G. A. R. Nobes and R. H. Marchessault (1999) in S. H. Imam, R. V. Greene, and B. R. Zaidi (Eds.), *Biopolymers: Utilizing Nature's Advanced Materials; ACS Symposium Series 723,* ACS, Washington, DC, pp. 110–145.
4. D. Jendrossek, A. Schirmer, and H. G. Schlegel (1996) *Appl. Microbiol. Biotechnol.* **46,** 451–463.

5. J. Mergaert, C. Anderson, A. Wouters, J. Swings, and K. Kersters (1992) *FEMS Microbiol. Rev.* **103,** 317–322.
6. K. Mukai, K. Yamada, and Y. Doi (1993) *Poly. Degrad. Stab.* **41,** 85–91.
7. T. Saito, M. Shiraki, T. Shimada, and M. Tatsumichi (1994) in Y. Doi and K. Fukuda (Eds.), *Biodegradable Plastics and Polymers*, Elsevier, Amsterdam, pp. 52–64.
8. J. Mergaert and J. Swings (1996) *J. Indus. Microbiol.* **17,** 463–469.
9. J. Mergaert, A. Wouters, C. Anderson, and J. Swings (1995) *Can. J. Microbiol.* **41,** 154–159.
10. T. Kobayashi, A. Sugiyama, Y. Kawase, T. Saito, J. Mergaert, and J. Swings (1999) *J. Environ. Polymer Degrad.* **7,** 9–18.
11. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (1990) *Current Protocols in Molecular Biology* **1,** 2.0.3–2.12.5.
12. M. T. Suzuki and S. J. Giovannoni (1996) *Appl. Environ. Microbiol.* **62,** 625–630.
13. S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman (1997) *Nucleic Acids Res.* **25,** 3389–3402.
14. S. H. Imam, S. H. Gordon, R. L. Shogren, T. R. Tosteson, N. S. Govind, and R. V. Greene (1999) *Appl. Environ. Microbiol.* **65,** 431–437.
15. E. Stackebrandt and B. M. Goebel (1994) *Int. J. Syst. Bacteriol.* **44,** 846–849.
16. G. Gauthier, M. Gauthier, and R. Christen (1995) *Int. J. Syst. Bacteriol.* **45,** 755–761.
17. C. Holmström and S. Kjelleberg (1999) *FEMS Microbiol. Ecol.* **30,** 285–293.